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Office of Pollution Prevention and Toxic Substances
USEPA Headquarters, 7201
Ariel Rios Building
1200 Pennsylvania Avenue, N. W.
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Re: Docket Number: Docket Control Number OPPTS-42212E
Issues Pertaining to the EPA's EDMVS --*In Utero*/Lactational Assay, Pubertal Male and Female Assays and 14-Day Intact Male Assay

Dear Dr. Vu:

The American Chemistry Council (ACC or the "Council") has played an active role in the development and implementation of the endocrine disruptor screening and testing program (EDSP) for several years.[1] The Council supports the Agency's establishment of the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) to provide technical advice and recommendations to EPA concerning the validation of endocrine disruptor screening and testing methods. ACC looks forward to the timely development and implementation of a scientifically sound EDSP.

The Council would like to bring to your attention the following issues pertaining to standardization and validation of proposed Tier 1 screening assays (the male and female pubertal assays and the 14-day intact male assay) and the proposed *in utero*/lactational assay.

***In Utero*/Lactational Protocol**

The case for an *in utero* protocol, separate from the Tier 2 mammalian multigeneration reproduction study, as part of an EDSP battery (e.g., Tier 1.5 or Tier 2), has not been established. With respect to the *in utero*/lactional assay, it appears that there is a perception that an *in utero* screen would provide some degree of increased sensitivity over mechanistic *in vivo* tier 1 screens

[1] The Council represents more than 90 percent of the productive capacity for basic industrial chemicals within the United States and its members are the leading companies engaged in the business of chemistry. EPA's endocrine disruptor screening and testing program (EDSP) may significantly affect the Council and its members. For that reason, the Council and its members have attempted to assist the Agency in developing and implementing its EDSP. In that regard, ACC and its members actively participated in EDSTAC and are actively participating in the EDMVS.

to detect substances which have the potential to interfere with the function of one or more components of the endocrine system. However, such preconceived benefits in sensitivity are not supported by available data, and overlook the critical design features of existing endocrine-specific screens (e.g., transcriptional activation, immature/castrate animal screens), which were purposely designed to maximize both sensitivity and specificity. Furthermore, the proposed study requirements for in-life and histopathology make the *in utero*/lactational study too long and too expensive to be practical for a Tier1 screen. EPA would be better served by focusing on endocrine-specific Tier 1 assays, so that Tier 1 yields accurate and readily interpretable results, using as few animals as possible. Furthermore, it is difficult to envision the circumstances under which the *in utero*/lactational assay would be used or triggered. Detailed comments are provided in Attachment 1.

Male and Female Pubertal Onset Assays

As defined by EDSTAC (EDSTAC, 1998) screens should be short-term, quick and inexpensive assays designed to detect specific hormonal activity (EDSTAC, 1998), and should be based on a discreet mode or mechanism of interaction with the endocrine system. Apical endpoint measurements, such as those in the male and female pubertal assays, which can be influenced by non-endocrine factors, are more appropriate for inclusion in definitive Tier 2 tests. The inclusion of numerous apical endpoints in these assays is problematic; as such apical endpoints are not specific indicators of a primary endocrine mechanism of action.

Dose selection for these assays is critical because there are many physiologic and toxicologic mechanisms that can affect pubertal onset. If the pubertal assays are required to use the Maximum Tolerated Dose (MTD), effects on body weight alone may will alter numerous endpoints and will likely affect the age at which pubertal onset occurs. When dose levels at or exceeding the MTD are used, as was the case in the studies EPA sponsored at TherImmune, interpretation of the results is very difficult and, therefore, conclusions regarding the robustness of the pubertal onset assays cannot be made at this time. Detailed comments are provided in Attachment 2.

14-Day Intact Male Assay

With respect to the Tier 1 screening battery, ACC urges EPA to undertake validation of the 14-Day Intact Male assay (see EDSTAC Report at 5-30). This is a mechanistic assay that satisfies the criteria for a Tier 1 screening method. It is a short-term, sensitive and specific assay that has been demonstrated to be capable of identifying substances which modulate the endocrine system, including compounds that have the potential to act as agonists or antagonists to the estrogen, androgen, progesterone, or dopamine receptor, 5α-reductase inhibitors, steroid biosynthesis inhibitors (aromatase and testosterone biosynthesis), and compounds that alter thyroid function. As indicated in Attachment 3, Table 1, there are many advantages of including this assay within the screening battery in lieu of the pubertal male assay and the Hershberger

assay. Evidence of this assay's sensitivity, specificity and utility for use as a Tier 1 screen is provided in numerous publications in the peer reviewed literature (Attachment 3).

The Council appreciates this opportunity to provide early input on matters related to the EDMVS. We look forward to working further with EPA and other interested parties on the validation of EPA's EDSP. Please don't hesitate to call me (703-741-5159) or Richard A. Becker, Ph.D., (703-741-5210) if you have questions.

Sincerely,

Original Signed By

Sarah H. Brozena
Deputy Co-Leader and Counsel
Public Health Team

Attachments

Attachment 1

Comments on the *In Utero*/Lactational Protocol Draft Review Paper

In preparation for discussions at the December meeting of the EDMVS, the EPA distributed a Draft Detailed Review Paper (DRP) on the *in utero*/lactational protocol. Overall, the DRP is very thorough and well referenced. ACC submits the following comments regarding technical aspects of the proposed study design(s):

Key Summary Points

- As acknowledged by the authors, we believe that this protocol is too ambitious to serve as a screening tool for the purpose described in the EDSTAC report. The study requirements for in-life and histopathology make the study too long and too expensive to be practical for the Tier 1 screening phase, and could significantly impact laboratory capacity for other testing programs currently underway.

- It is difficult to envision that this study would truly replace other, more specific study designs in the Tier 1 screening program. As noted by the authors, some endocrine-sensitive endpoints can also be altered by non-endocrine mechanisms. It is likely that moderately toxic chemicals could have equivocal results requiring that more specific screens be conducted to sort out the findings. This will not only increase animal usage, but also slow down the screening program. We agree with the authors that this study design may be more appropriate for consideration after the mechanistic Tier 1 screens have been conducted. However, EPA would need to provide guidance for when this would be appropriate and clearly define how the results of such an assay would be used.

- A stated purpose of the *in utero*/lactational study, to "fully evaluate effects on subsequent growth and development," is incompatible with the goal of Tier 1 screening, i.e., to identify chemicals with <u>potential</u> to interfere with the function of the endocrine system. Full evaluation of adverse effects is the goal of Tier 2 testing. The DRP has not provided examples of chemicals for which the simpler, faster, mechanistic Tier 1 screens would not detect <u>potential</u>.

- Although the *in utero*/lactational assay may initially appear of some interest, when the implementation issues are considered, it's usefulness is questionable. It would be very difficult to define the circumstances under which it would be used or triggered. Secondly, such an assay would not be sufficiently definitive to permit a clear conclusion. It would seem better to have a robust Tier I battery, and, if Tier II is triggered, then to conduct the appropriate definitive test.

The following are comments to specific sections or statements in the DRP. Since the stated purpose of the DRP is to investigate the status of various proposed protocols that incorporate *in utero*/lactational exposure for detection of endocrine disruptors for EPA application, we have focused our comments toward this goal.

Executive Summary

The statement is made that "Since effects associated with endocrine disruption may be latent or may not appear until maturation of the reproductive systems, Tier 1 screening must include an evaluation of the endocrine-disrupting activity of the test compound on the postnatal development and maturation of the mammalian reproductive tract….any mammalian assay or test must include exposure to the test compound *in utero* and during lactation, in order to fully evaluate effects on subsequent growth and development." However, the purpose of Tier 1 screening is not to fully evaluate any effects from endocrine disruption, but to detect potential for endocrine activity. Full evaluation will require the resources, most importantly time, animal group sizes, and technical competence, that is currently incorporated into the Tier 2 testing program.

2.4 Objective of the *In Utero*/Lactational Protocol within the EDSP

Here, the DRP addresses the differing views within EDSTAC as to "whether there is scientific evidence of known endocrine disruptors or reproductive toxicants that can affect the prenatal stage of development without affecting the adult or prematuration stages, and whether effective doses and affected endpoints may differ among the three life stages." While there is a common preconception that *in utero* exposures result in the

greatest sensitivity, the DRP does not provide data to support this contention. It is important to recognize that Tier I contains several specialized tests designed to maximize sensitivity, such as the receptor binding / gene transcription assays which probe activity on the molecular level, and the uterotrophic and Hershberger assays which use immature and/or castrated animals to heighten sensitivity. An *in utero* assay with 10 litters per group and more apical end points is unlikely to match the sensitivity of the highly specific Tier I screens. Furthermore, there should be ample opportunity to evaluate relative sensitivity of developing and adult organisms in Tier 2 testing programs.

The DRP also notes that a validated *in utero* assay should be evaluated for its potential to replace one or more of the recommended Tier 1 assays and its overall impact to the cost effectiveness of the Tier 1 battery. In our view, overall impact should include not only cost, but also animal usage, laboratory resources, and time. It is difficult to believe that results of an *in utero* assay which indicate no effects upon the developing or adult organism would be considered a rigorous test of hazard potential. In such a case the likely outcome would be to follow up this assay with the recommended Tier 1 battery. Secondly, it is likely that some proportion of chemicals with the potential for systemic toxicity could impact one or more endocrine sensitive endpoints that are also sensitive to generalized toxicity. In this scenario, the testing sponsors would have the option of returning to the Tier 1 level to conduct the recommended screens for mechanistic data or to conduct full guideline apical studies for Tier 2 testing. Either of these scenarios would increase animal usage and the use of laboratory resources. The overall impact would be to increase the time required to screen a chemical. It is our perspective that the only chemicals for which this assay would potentially reduce animal usage would be for rather potent endocrine disruptors with clear effects upon endocrine-sensitive endpoints in the absence of systemic findings.

3.4 Appropriateness of Endpoints for Measuring the Endocrine Disruption after *In Utero*/Lactational Exposure

The DRP clearly points out several important considerations critical to selection of testing endpoints, including reproducibility, sensitivity, and relevance. The specific endpoints noted in this section, such as anogenital distance, nipple retention in males, onset of puberty, and others, are included and rigorously evaluated (or will be) in Tier 2 tests. Importantly, the statistical sensitivity to avoid false positives and false negatives is incorporated into the guideline protocols. The proposed *in utero*/lactational assay would utilize 10-20 animals/exposure level, which would either be short of the necessary statistical sensitivity or result in substantial use of animals.

The validation of the endpoints incorporated into this assay would provide valuable information toward Tier 2 test result interpretation. The authors note the importance of interpretation of study results throughout this section.

3.5.2 Estrogen Modulators, Bisphenol A

Our only comment to this section relates to the data listed in the text for anogenital distance provided by the U.S. EPA (1998b). It is not clear how an increase of 0.04 mm can be found between group values of 0.98 mm and 0.99 mm.

4.0 Candidate Protocols

We agree with the DRP that assessment through pubertal development is necessary to fully evaluate endocrine-mediated effects upon reproduction. Both Protocols B and C provide that period of evaluation. Evaluation of adverse effects upon all phases of reproduction is the basis of the OPPTS 870.3800 Reproduction and Fertility Effects testing guideline. Validation of the newer endpoints of this two-generation reproduction study is a component of Tier 2 validation efforts.

Section 4.2.5.1 makes the statement that we believe most succinctly summarizes our opinion: "However, the study design may be too ambitious to serve as a screening study." We believe that a study design utilizing 40 - 120 adult animals (plus 560-14,400 offspring, assuming an average litter size of 14), 10 weeks of in-life, and histopathology is simply too much for initial screening of any material. We also believe that this study, if validated for sensitivity, reproducibility, and robustness, might be judiciously

incorporated into the Tier 2 testing of select materials (how would these be chosen, what purpose would the data serve?), as well as provide valuable background information for the Tier 2 program.

Minor notes to the protocol proposals:

Section 4.2.3.4: Doses Used "…be relevant to human/wildlife exposure levels for testing." Although limit doses are specified in several EPA testing guidelines, it would be unique for relevant exposure to be a factor in dose selection for EPA testing programs. It may also be that for many chemicals the relevant exposure levels are unknown at the time of testing.

Section 4.4.4.13: Decision Criteria Used to Classify a Test Chemical:

We presume that by classification the DRP is actually referring to the prioritization of the test chemical, since the Tier 1 screening program is designed to catch chemicals with *potential* for endocrine disruption, but inadequate to classify them as such.

Section 5.2 Test Chemicals Applicable to Assay:

We agree that this assay may be inappropriate for any suspected chemical/dose-level that causes overt maternal toxicity or interferes with nutritional status. We also agree that compounds that cross the placenta and/or are secreted into breast milk could be used in this assay *after* Tier 1, if applicable. As a practical matter, it is highly unlikely that the pharmacokinetic behavior of a chemical would be known at the time of screening and testing.

<u>Section 6.3.1 Advantages/Disadvantages of the Endocrine Disruptor Assays Related to Animal Use</u>

The use and humane care of laboratory animals is an important factor in industry testing programs. We view it as an important consideration in the design of Tier 1 and Tier 2 programs. We question the DRP statement that "By using this screen in conjunction with the other Tier 1 screens, there is a much better chance of identifying compounds that need to be tested, and reducing the overall number of animals that must be used to identify these compounds". The number of animals used in the in utero protocol is quite high (40 dams x 14 pups/litter = 560 animals per compound), and given the questionable benefits of such a protocol, it is likely to result in *increased* animal use, rather than reduced animal use.

Conclusion

The authors of the DRP have done a thorough job of presenting three separate protocols and discussing their potential advantages and disadvantages. However, we believe the case for an in utero protocol as part of an EDSP screening battery (e.g., Tier 1.5 or Tier 2) has not been established. The preconceived benefits in sensitivity are not supported by available data, and overlook the critical design features of existing endocrine-specific screens (e.g., transcriptional activation, immature/castrate animal screens) which were purposely designed to maximize both sensitivity and specificity. EPA would be better served by focusing on endocrine-specific Tier I assays, so that Tier I yields accurate and readily interpretable results, using as few animals as possible.

Attachment 2
Comments on the Male and Female Pubertal Onset Assays

Evaluation of Male and Female Pubertal Onset Assays

In preparation for discussion of the male and female pubertal onset assays at the December meeting of the EDMVS, the following documents were distributed by EPA: 1) TherImmune Research Reports: Assessment of Pubertal Development and Thyroid Function in Juvenile Male and Female Rats [Blocks 1 and 2]; 2) Study Summary on the Assessment of Pubertal Development and Thyroid Function in Juvenile Male and Female Rats (EPA Requisition/Reference No.: AC5001 QT-RT-99-002276); and 3) Two articles from *Critical Reviews in Toxicology* that review the available data on puberty onset in male (Stoker *et al*., 2000a) and female (Goldman *et al*., 2000) rats and outline protocols to conduct these assays. Each of these documents is discussed briefly with emphasis on their relevance to validation and standardization of the male and female pubertal onset assays for endocrine disrupter Tier I screening.

Key Summary Points

- **Dose selection is critical. If studies are required to use the Maximum Tolerated Dose (MTD), effects on body weight will alter numerous assay endpoints (e.g., epididymidal, prostate/ventral prostate and seminal vesicle weights), and may affect the age at which pubertal onset occurs. Furthermore, estrogenic and anti-thyroid agents may decrease the rate of growth, making it difficult to discern endocrine effects from systemic toxicity.**

- **There is inherent inter-animal variability in the age at which puberty is achieved. This contributes to the debate as to interpretation of small (i.e., <2 days) changes in age at pubertal onset. During preputial examinations, a persistent preputial thread of tissue has been noted infrequently in some animals. This thread can occur in the absence of treatment and complicates the determination of age at which preputial separation is complete.**

- **The TherImmune pubertal onset studies exceeded the MTD or used dose levels 2-10X the dose levels used by other investigators to detect endocrine activity. Due to persistent preputial threads, mean age at pubertal onset in control Long-Evans rats varied from 44.8 to 50.2 days of age. Furthermore, there was variability ($\geq$25%) between block 1 and block 2 control values for pituitary, adrenal, seminal vesicle and ovarian weights. Uterine weights may be highly variable due to estrous cycle stage at the time of necropsy.**

- **Two review articles on the pubertal onset assays (*Critical Review in. Toxicology*) document the considerable number of agents that can affect pubertal onset, illustrating the apical nature of this assay and the difficulty of identifying a mechanism of action for positive agents.**

- **Protocol descriptions in these review articles require that time-mated animals are used to select weanlings for pubertal assays. Litter effect is not controlled in these studies. Ordering litters of pups would be more efficient and prevent excess animal usage. Liver, kidney, pituitary and adrenal weights are listed as required end points in the female assay, but designated as optional end points in EDSTAC and in the male assay. None of these organ weights were critical for the identification of endocrine activity in the TherImmune studies.**

- **Consideration should be given to examining the dose-response relationship for weaker endocrine active agents, including aromatase inhibitors and anti-thyroid agents.**

1) TherImmune Research reports: Assessment of Pubertal Development and Thyroid Function in Juvenile Male and Female Rats (Blocks 1 and 2)

As outlined by the EPA, the goals of the male and female pubertal onset studies conducted by TherImmune were identified as follows: 1) to provide a preliminary validation of the protocols for the assessment of pubertal development and thyroid function in juvenile male and female rats; 2) to assess the robustness of the protocols with regard to intra-laboratory and inter-strain sources of variation; and 3) to provide documentation of the operating procedures required to successfully implement the protocols.

An evaluation of the data provided by TherImmune suggests that these goals were not met. Overall, the TherImmune studies did not provide suitable validation data for the assessment of the pubertal onset assays. The maximum tolerated dose (MTD; dose that produces an approximately 10% change in body weight gain below control animals without clinical signs of toxicity) was exceeded in most experiments (i.e., 5 of 12 experiments using Sprague-Dawley rats, 11 of 12 experiments using Long-Evans rats). Furthermore, the robustness of the protocols was not established for either intra-laboratory nor inter-strain sources of variation. Because the selected dose levels exceeded the MTD, conclusions regarding the robustness of the pubertal onset assays cannot be made. In cases where the MTD was not exceeded (e.g., Table 2), the ability to detect potent agents at high concentrations is weak evidence of the reliability of these assays. Greater credibility can be gained from the published reports of other investigators who obtained similar results with these test materials at 2-10X lower dose levels, despite using non-standardized protocols (see Table 2).

It should be recognized that the studies conducted at TherImmune included several endpoints that are optional when conducting pubertal onset assays (EDSTAC, 1998). These endpoints include liver, kidney, pituitary and adrenal weights. None of these organ weights were important contributors to the identification of endocrine-active compounds in this study; thus, at this stage, these endpoints should remain optional.

Results using Long-Evans rats:

In the experiments using male and female Long-Evans rats, the MTD was exceeded in all cases (see Tables 1a and b), making interpretation of these data very difficult. Changes in body

weight (10-15%) have been shown to impact the age at pubertal onset (Ashby and Lefevre, 2000; Stoker *et al*., 2000a; Goldman *et al*., 2000). Data suggest that body weight and pubertal onset function as a continuum, so sufficient changes in body weight will impact age at pubertal onset. Data from feed restriction studies (O'Connor *et al*., 2000; Stoker *et al*., 2000b; Marty *et al*., 2001) have demonstrated that epididymal, seminal vesicle and prostate/ventral prostate weights are altered by effects on body weight.

Also contributing to the complexity of pubertal assay results is the natural variability that is inherent in age at puberty onset. Using Long-Evans rats, the mean age at preputial separation varied from 44.83 days of age in block 1 to 50.17 days of age in block 2. It was reported that these rats had a persistent thread of tissue between the glans penis and the prepuce that delayed complete preputial separation. The authors cite biological variability as a likely explanation for this finding. Although infrequent, other laboratories (including Dow) also have reported a persistent thread of tissue in some animals when conducting preputial examinations (personal communication). Classifying these animals as "not separated" can result in a bimodal distribution of age at preputial separation.

Results using Sprague-Dawley rats:

In the experiments using the Sprague-Dawley rats, propylthiouracil and pimozide exceeded the MTD, inducing approximately 55 and 18% decreases in body weight, respectively; however, not all treatments exceeded the MTD. Some compounds (Table 2) were detected as endocrine active at doses that did not produce a 10% decrement in terminal body weight. In many cases, there are published reports available using these compounds in pubertal assays or assessing pubertal assay endpoints (e.g., flutamide, ketoconazole, dibutylphthalate, ethynyl estradiol, and methoxychlor). However, in the present studies, these compounds were administered at doses 2-10X the dose level used by other investigators.

For the various materials tested, there are additional observations that should be noted. They are as follows:

Flutamide: Flutamide was detected at doses that were 4X the level used by Ashby and Lefevre (2000) and 10X the level used by Yamada *et al.* (2000). Each investigator detected significant changes in epididymal, ventral prostate and seminal vesicle weights (see Table 2).

Methyl Testosterone: Typically, androgenic substances increase body mass and thereby, increase body weight. Methyl testosterone, administered to intact, juvenile male rats increased body weight (in some cases significantly) until approximately 38 days of age. Thereafter, testosterone-treated animals had lower body weights than control animals with statistically identified decreases in body weight noted by the end of the study. Preputial separation in these animals occurred at 37.3 days of age; thus, the change in body weight relative to controls is consistent with anabolic effects prior to maturation of the hypothalamic-pituitary-gonadal (HPG) axis followed by inhibition of the HPG axis (negative feedback) as maturation progressed. This hypothesis is substantiated by decreased testes weights (blocks 1 and 2) and lower epididymal (block 1) or pituitary (block 2) weights. Testicular histopathology (hypospermatogenesis, hypospermia and interstitial cell atrophy) was observed.

The effects of methyl testosterone on seminal vesicle weight exemplify the variability in these measurements that was identified by the EPA summary. In block 1, seminal vesicle weights were increased by 30.8%, whereas in block 2, the same concentration of methyl testosterone increased seminal vesicle weights by 82.8%. This finding is partly attributable to a 25% weight differential in control seminal vesicle values (0.548 vs. 0.406).

Propylthiouracil (PTU): Results for this compound were similar to previously reported findings (Marty *et al*., 1999; 2001c). Animals were rendered extremely hypothyroid by treatment, which altered numerous endpoints secondary to dramatic effects on growth. Thyroid histology, T4 and TSH levels were significantly altered. It is noteworthy that PTU is a strong goitrogen. Consideration should be given to weaker thyroid agents. For example, the thyroid effects of phenobarbital, a weak thyroid active agent, were detected primarily due to altered thyroid weight (Marty *et al*., 2001c), which is not a required endpoint of pubertal onset assays.

Ketoconazole: In the male pubertal onset assay, ketoconazole was detected at doses 4X greater than the doses used by other investigators (see Table 2). Use of these high dose levels does not adequately test the robustness of the assay.

In the female assay, ketoconazole produced an increase in ovarian weight (40.9%) in block 1 and a decrease in ovarian weight (10.9%) in block 2. Uterine weights were decreased in

both blocks (26.9 and 30.2% decreases; the 30.2% decrease was statistically identified). Ovarian weights varied by 25% in control animals (0.0615 g in block 1 vs. 0.046 g in block 2).

Pimozide: Although clinical signs were not observed during the TherImmune studies, dopamine antagonists are often associated with altered ambulatory behavior. In the case of pimozide, several reports cite decreased locomotor activity (Lambert and Porter, 1992; Agmo and Soria, 1999; Sousa *et al*., 2001). Are overt signs of neurotoxicity sufficient to constitute an MTD? What interpretation is given to Tier I pubertal assays if endocrine effects are detected only in the presence of overt neurotoxic signs?

Dibutylphthalate (DBP): Results for DBP were variable in this assay. Within the two blocks, a 28% decrease in seminal vesicle weights in block 1 was the only reproductive/ASG weight statistically identified, despite using 2X the dose used by Ashby and Lefevre (2000) (see Table 2). Interestingly, 13 and 30% decreases in testes weight were not identified statistically within the blocks.

Ethynyl Estradiol[2] *(EE):* As mentioned for estrogenic compounds, EE produced significant decreases in terminal body weight (see Table 3), indicating that greater guidance is required for dose selection for estrogenic compounds. Vaginal opening occurred at a younger age and a lower body weight, effects consistent with estrogenic agents. Despite 19% decreases in ovarian weights in blocks 1 and 2, neither ovarian nor uterine weights were significantly affected with EE treatment.

Methoxychlor[1]*:* As with EE, methoxychlor induced >10% decrement in body weight in both test blocks. Similarly, vaginal opening was accelerated. Neither uterine nor ovarian weights were significantly altered with methoxychlor treatment. Uterine weights were increased in block 1 (15.4%) and decreased in block 2 (27.9%). This example highlights the difficulty in collecting uterine weights in cycling animals.

Dose level selection:

As was pointed out by the authors of the EPA Summary (#2 below), dose level selection may be difficult for estrogenic compounds, which may alter feed consumption and decrease the

rate of growth. This also applies to antithyroid agents, where reduced thyroid function also may affect growth.

2) Study Summary on the Assessment of Pubertal Development and Thyroid Function in Juvenile Male and Female Rats (EPA Requisition/Reference No.: AC5001 QT-RT-99-002276

Technical monitors at the E.P.A. have reviewed and commented on the TherImmune reports. Many of the shortfalls discussed above were identified in the E.P.A. report too, including the variation in the ages of preputial separation in Long-Evans rats and the large degree of variability in fluid-filled (and small) tissue weights. Once again, the small tissues cited in this report (adrenals and pituitary) are optional endpoints in pubertal assays. The difficulty in identifying appropriate dose selection criteria has been mentioned previously, particularly for estrogenic and thyroid-active compounds that can affect rate of growth.

In their discussion of additional issues, the E.P.A. suggested that MANCOVA be deleted and ANCOVA be conducted, allowing for necropsy body weight to serve as a covariate. This is not the best use of ANCOVA analysis, because terminal body weight also is affected by treatment. The covariate should be independent of treatment (e.g., weanling weight).

With regard to establishing performance criteria, this proposal would be valuable once the natural variation inherent in the various endpoints has been defined and well documented. Performance criteria should be derived from multiple laboratories in a study of sufficient magnitude so that variations can be detected. Without understanding the basis for the variation, the E.P.A. sentiment that "the laboratory should be able to demonstrate the ability to detect the expected effects for all endpoints using positive controls" may be difficult to accomplish routinely.

Additional experiments to examine the dose-response relationship for these compounds and other weaker endocrine-active agents (e.g., aromatase inhibitors, weak thyroid agents, etc.) are critical for the evaluation of these assays. Intra-and inter-laboratory variations in the lower limits of detection should be examined. In these dose-response studies, additional compounds having both endocrine and non-endocrine activities should be tested.

3) Review articles from *Critical Reviews in Toxicology* that discuss male (Stoker *et al*., 2000) and female (Goldman *et al*., 2000) pubertal onset.

Included in the information under consideration were copies of the male and female pubertal assay reviews which appeared in *Critical Reviews in Toxicology* (Stoker *et al*., 2000; Goldman *et al*., 2000). These reviews are comprehensive and reasonably well done. Because of this, these reviews emphasize the apical nature of puberty onset. Within each article is a fairly inclusive table listing agents that have been shown to alter puberty onset. These tables (Tables 4, 6 and 7 in Goldman *et al*., 2000; Table 3 in Stoker *et al*., 2000a) illustrate not only the

[1]Ovarian and uterine organ weights were not sensitive indicators of endocrine activity in the TherImmune studies. Neither ethynyl estradiol nor methoxychlor significantly affected these organ weights. Ketoconazole significantly decreased uterine weight in block 2, but this decrease was not significantly identified in block 1 (see Table 3).

considerable number of agents that can affect puberty onset, but also the diversity of chemical and biological activities that can impact this event. Numerous agents that do not directly target the endocrine system or alter endocrine function secondary to another toxic event (e.g., neurotoxicants operating at higher brain centers) will be identified as endocrine active agents and subject to Tier II testing. Because of the apical nature of puberty onset, the specificity of these assays must be verified. Furthermore, for chemicals classified as positive in the pubertal onset assays, identification of a mechanism of action may not be possible.

The articles by Stoker and Goldman also describe critical aspects of the male and female pubertal onset assays. According to both protocols, pubertal assays begin with either in-house mating or time-mated female rats (gestation day 7-10 arrival). This protocol could result in increased animal usage if only one pubertal assay (male or female) is conducted, because pups of the opposite sex would be discarded. At weaning, animals are randomized into groups (15 animals /group) based on body weight, using only animals whose body weights are $\pm$ 2SD from the mean ($\pm$ 8 g is typical at the E.P.A.). At termination, body weight at necropsy can be used as a covariate for organ weight data, although this is not ideal because the covariate should be independent of treatment. According to Goldman *et al*. (2000), liver, kidney, pituitary and adrenal weights are required end points in the female pubertal onset assay, although they are listed as optional endpoints in the EDSTAC document. In Stoker *et al.* (2000a), the EDSTAC-recommended exposure period of 20 days was extended to 30 days from postnatal day 23-53.

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Table 1a: Terminal Body Weight Differences in Male Long-Evans Rats With Various Treatments (TherImmune Studies)

lock	control	lutamide	Methyl Testosterone	Propylthiouracil	Ketoconazole	imozide	Dibutylphthalate
	93.3 g	1.3% ↓	10.4% ↓	62.4% ↓	3.3% ↓	5.3% ↓	10.8% ↓
	75.5 g	.6% ↓	9.8% ↓	61.8% ↓	0.8% ↓	2.5% ↓	16.1% ↓

Table 1b: Terminal Body Weight Differences in Female Long-Evans Rats With Various Treatments (TherImmune Studies)

lock	control	Ethynyl Estradiol	Tamoxifen	Propylthiouracil	Ketoconazole	imozide	Methoxychlor
	64.7 g	12.5% ↓	2 1.6% ↓	45.1% ↓	8.9% ↓	4.9% ↓	14.6% ↓
	155.4g*	10.5% ↓	2 1.4% ↓	48.2% ↓	1 3.5% ↓	1.2% ↓	11.3% ↓

***Estimated values based on pnd 42 body weights, because not all terminal body weights appear in the data set.**

Table 2: A Comparison of the TherImmune Results for the Male Pubertal Onset Assay with Other Published Reports

	Flutamide				Dibutyl Phthalate (DBP)			Ketoconazole			
Dose	100 mkd	100 mkd	25 mkd	10 mkd	1000 mkd	1000 mkd	500 mkd	100 mkd	100 mkd	25 mkd	24 mkd
Investigator	Ther-Immune (Block 1)	Ther-Immune (Block 2)	Ashby and Lefevre, 2000	Yamada *et al*., 2000	Ther-Immune (Block 1)	Ther-Immune (Block 2)	Ashby and Lefevre, 2000	Ther-Immune (Block 1)	Ther-Immune (Block 2)	Ashby and Lefevre, 2000	Marty *et al*., 2001b
	Percentage Difference From Respective Controls.										
Terminal	1.5% ↓	6.7% ↓	1.4% ↑	NS	5.1% ↓	6.7% ↓	1.3% ↑	5.8% ↓	**10.0%** ↓	0.3% ↑	4.5% ↓
Testes	**19.8%** ↑	7.8% ↑	0%	0%	12.9% ↓	29.6% ↓	6.9% ↓	6.2% ↓	4.4% ↓	0%	0.7% ↓
Epididymides	**43.6%** ↓	**31.1%** ↓	**32.7%** ↓	**32.0%** ↓	7.5% ↓	3.1% ↓	**10.1%** ↓	**12.9%** ↓	8.6% ↓	0.6% ↓	**17.4%** ↓
Ventral	**52.0%** ↓	**81.7%** ↓	**38.5%** ↓	**31.6%** ↓	4.5% ↓	11.9% ↓	9.1% ↓	**38.6%** ↓	**37.4%** ↓	**17.7%** ↓	NA
Seminal Ves.	**84.3%** ↓	**83.7%** ↓	**59.1%** ↓	**65.2%** ↓	**28.3%** ↓	8.6% ↓	**22.8%** ↓	**50.4%** ↓	**38.4%** ↓	**19.7%** ↓	NA
LABC	**61.5%** ↓	**53.5%** ↓	NA	**27.5%** ↓	15.0% ↓	20.4% ↓	NA	20.8% ↓	28.5%	NA	NA
Age at PPS	**+11.5**	**+11.5**	**~+5.9**	NA	+2.0 days	+0.5 days	+1.9 days	**+3.8**	**+2.8**	-0.3 days	+1.7 days
BWt at PPS	38.0% ↑	23.4% ↑	NA	NA	1.9% ↓	0.27% ↓	**9.1%** ↑	**7.3%** ↑	1.2% ↑	2.1% ↓	3.3% ↓

Table 3: A Comparison of the TherImmune Results for the Female Pubertal Onset Assay with Other Published Reports

	Ethinyl Estradiol			Methoxychlor			Ketoconazole		
Dose	0.005 mkd	0.005 mkd	0.01 mkd	100 mkd	100 mkd	50 mkd	100 mkd	100 mkd	100 mkd
Investigator	Ther-Immune (Block 1)	Ther-Immune (Block 2)	Laws *et al*., 2000	Ther-Immune (Block 1)	Ther-Immune (Block 2)	Ashby and Lefevre, 2000	Ther-Immune (Block 1)	Ther-Immune (Block 2)	Marty *et al*., 1999
	Percentage Difference From Respective Controls.								
Terminal	**6.0%** ↓	NA	2.6% ↓	**6.4%** ↓	NA	4.8% ↓	2.4% ↓	NA	7.3% ↓
Ovaries	19.0% ↓	19.6% ↓		20.6% ↓	10.9% ↓		40.9% ↑	10.9% ↓	29.3% ↓
Uterus	0%	5.9% ↓		15.4% ↑	27.9% ↓		26.9% ↓	**30.2%** ↓	**45.0%** ↓
Age at VO	**-8.3 days**	**-9.5 days**	**-6.0 days**	**-8.0 days**	**-8.7 days**	**-8.4 days**	+1.8 days	**+3.2**	**+6.2**
BWt at VO	**37.4%** ↓	**42.6%** ↓		**37.0%** ↓	**40.3%** ↓		5.4% ↑	5.4% ↑	**16.4%** ↑

NA – not available; terminal body weight not given for all animals.

¤ Body weight at 33 days of age, not terminal body weight.

Attachment 3
Intact Male Assay – An Alternate Tier 1 Screening Assay

In the EDSTAC report (1998), the Intact Male Assay is envisioned as replacing the Hershberger, female pubertal, *in vitro* steroidogenesis, and placental aromatase assays. The Intact Male assay is the result of over eleven years of work to develop short-duration *in vivo* screening methods to identify endocrine modes of action. (Cook *et al.* 1997). The Intact Male Assay is a mechanistic assay that satisfies the criteria for a Tier 1 screening method. It is a short-term, sensitive and specific assay that has been demonstrated to be capable of identifying substances which modulate the endocrine system, including compounds that have the potential to act as agonists or antagonists to the estrogen, androgen, progesterone, or dopamine receptor, 5α-reductase inhibitors, steroid biosynthesis inhibitors (aromatase and testosterone biosynthesis), and compounds that alter thyroid function. As indicated in Table 1, there are many advantages of including this assay within the screening battery in lieu of the pubertal male assay and the Hershberger assay. Evidence of this assay's sensitivity, specificity and utility for use as a Tier 1 screen is provided in numerous publications in the peer reviewed literature. ACC urges EPA to undertake validation of the Intact Male assay as part of its efforts to standardize and validate EDTAC recommended Tier 1 screens.

The Intact Male assay is designed to be run in parallel with the uterotrophic assay and the *in vitro* receptor binding and/or transcriptional activation assays, and actually can identify a broader spectrum of hormonally active substances than proposed by EDSTAC. It is designed to identify compounds that have the potential to act as agonists or antagonists to the estrogen receptor, androgen receptor or progesterone receptor, dopamine modulators, steroid biosynthesis inhibitors (aromatase, 5α-reductase, and testosterone biosynthesis), or compounds that alter thyroid function . The 15-day Intact Male battery combines organ weight measurements, a comprehensive hormonal battery, and limited histopathology to achieve these goals. It provides specific information on the mode of action of a compound by establishing a "fingerprint" of changes in the endpoints to identify hormonally active substances.

TABLE 1. Comparison of EDSTAC Alternate Batteries 1 and 2 (Mammalian Assays Only)

Characteristic	Alternate Battery 1	Alternate Battery 2
Assays included	**15-day intact male** Uterotrophic Receptor binding/transactivation	**Pubertal male** Uterotrophic Receptor binding/transactivation
Performance[a]	7/7 correctly identified positives, 1 equivocal (due to strain differences)	6/7 correctly identified positives 1 equivocal (due to interlaboratory variation), 1 false negative (aromatase inhibitor)
Interpretability	Based on established "fingerprints" of specific endocrine activities	Can be difficult due to reliance on apical end points (especially for organ weight decreases)
Specificity	Enhanced due to hormonal end points	Lower due to use of reproductive end points that can be affected by non-endocrine mechanisms
Technical difficulty	Higher due to hormone analyses	Lower due to use of routine end points
No. animals per compound screened[b]	**96** (36 for uterotrophic assay) (60 for intact male assay)	**156** (36 for uterotrophic assay) (60 for pubertal male assay) (approx. 60 unused female weanlings)
Cost	Can be high if hormone analyses are contracted, lower if done in-house	Similar to Battery 1 with in-house hormone analysis

[a] Based on data for compounds tested by the same route in both the 15-day intact male and male pubertal assays (adapted from O'Connor et al., submitted). Does not include new, unpublished data.

KEY POINTS

- 15-day male end points are more specific for endocrine mode-of-action vs. apical end points in the pubertal assays.
- 15-day male assay uses fewer total animals vs. pubertal male (hidden loss of female littermates not used in assay)
- Criteria for establishing a positive in the pubertal assay will be more difficult.
- Specificity of pubertal assays is a concern, since puberty is influenced by body weight, diet, etc.

The study design and rationale for the Intact Male assay have been described (Cook *et al.* 1997). Male rats are dosed daily for 15 days with the test compound and euthanized on the morning of test day 15, approximately 2 hours after the last administered dose. At the terminal euthanization, the liver, thyroid gland, and reproductive organs [testes, epididymides, prostate, seminal vesicles with fluid, accessory sex gland unit (ASG; composed of the prostate, seminal vesicles with fluid, and coagulating glands)] are weighed, and the testes, epididymides, and thyroid gland are saved for histopathological evaluation. Blood is collected and serum is prepared for hormonal evaluation (testosterone, estradiol, DHT, LH, FSH, prolactin, T_3, T_4, TSH). By comparing the "fingerprint" of the organ weight and hormonal endpoints for an unknown compound to a series of positive controls, the 15-day Intact Male battery not only identifies potential hormonally active agents, but aids in the characterization of their mode of action.

O'Connor and co-workers recently completed a pre-validation exercise for an integrated Tier I testing strategy (O'Connor *et al.* 1996, 1998a,b, 1999a,b, 2000a,b). The two primary goals of the pre-validation exercise were to test the hypothesis that distinct "fingerprints" could be identified for each type of endocrine activity, and to determine which of the endpoints evaluated in the pre-validation exercise should be included in a final screen. By developing a "fingerprint" for each type of endocrine activity, the pattern of the responses for compounds with unknown endocrine activity can be compared to those from the positive controls. To accomplish these goals, 15 positive controls with known endocrine activities were examined in an integrated Tier I screening battery consisting of the 5-day uterotrophic assay, 15-day Intact Male assay, and *in vitro* YTS (O'Connor *et al.* 1996, 1998a,b, 1999a,b, 2000a,b). Each endpoint was evaluated for the variability, stability over time, predictiveness, and dose-dependency for each of the positive endocrine controls.

The research efforts to develop and standardize the Intact Male assay have resulted in a sufficient body of scientific data that demonstrates the assays ability to confidently identify hormonally active substances. ACC urges EPA to undertake validation of the Intact Male assay as part of its efforts to standardize and validate EDTAC recommended Tier 1 screens.

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